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Excellent excrement? Frass impacts on a soil's microbial community, processes and metal bioavailability

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ABSTRACT

The commercial rearing of insects is a growing economic sector. Therefore, an assessment was made of the potential of its by-product, frass, to be a soil improver. Essential plant nutrients were extracted (using 0.01 M $CaCl_2$ or Mehlich 3) from frass of mealworms (*Tenebrio molitor*), black soldier flies (*Hermetia illucens*) and buffalo worms (*Alphitobius diaperinus*). A 28-day incubation, in which frass was added to a sandy loam soil at application rates of 2.5% or 5% (w/w), assessed its effects on soil microbial biomass, abundance of bacteria, archaea and fungi, carbon mineralisation and nitrification. In a separate 56-day incubation, the impact of frass on heavy metal bioavailability in an artificially contaminated, carbon-poor substrate was tested. All frass types featured high electrical conductivity, a mildly acidic to neutral pH and C: N ratios between 11 and 16. Black soldier fly frass (BSFF) was richer in extractable ammonium, phosphorus, potassium and magnesium than mealworm frass (MWF) and buffalo worm frass (BWF) but poorer in extractable calcium. All frass types stimulated carbon mineralisation, nitrification, bacterial and archaeal 16S rRNA gene copy numbers, and fungal biomass as determined by ergosterol concentrations. Bacterial and particularly fungal abundances were stimulated by the 5% frass application rate whereas archaeal abundances were greater in the 2.5% application rate regimes. The 2.5% application rate of MWF and BWF led to a profound build-up of soil extractable nitrite. Correspondingly, these treatments featured the highest 16S rRNA gene copy numbers of archaea, a domain encompassing organisms which oxidise ammonium to nitrite. No nitrite was detectable in soil amended with BSFF. The 5% application rates induced microbial biomass growth (as determined by extractable DNA concentrations) only when BSFF was applied. This was possibly due to differences in the frass types' extractable nutrient or labile carbon contents. BSFF and BWF amendment led to significantly higher microbial biomass in a metal-contaminated substrate. This was likely due to frass providing nutrients, energy and reduced metal bioavailability: extractable zinc, copper, cadmium and nickel concentrations fell due to increased metal sorption and complexation. All frass types could be used as ameliorants in metal-contaminated soils, while BSFF shows most promise as an organic fertiliser as its use did not cause soil nitrite build-up.

1. Introduction

Anthropogenic activities such as the intensification of animal husbandry can result in excess nutrients being delivered to soils and water bodies if, for example, the plant demand for nutrients is exceeded by that supplied via manure application. Against this backdrop, it is desirable to support nutrient recycling and connect agro-food-waste streams locally (van der Wiel et al., 2019). A rather novel method of using wastes, simultaneously reducing the aforementioned nutrient excess and generating commercially viable products, is the rearing of insects. This sector is growing worldwide as insects represent an environmentally sound alternative animal feedstock (Oonincx et al., 2015), food (Yang et al., 2019) or lipid and protein source (Gold et al., 2018). Insect larvae of mealworms (*Tenebrio molitor*), black soldier flies (*Hermetia illucens*) and buffalo worms (*Alphitobius diaperinus*) are capable of utilising diverse organic feed sources. Mealworms and buffalo worms can be reared using by-products from potato processing, bakeries, breweries and bioethanol production (van Broekhoven et al., 2015). Black soldier flies have been reported to consume manures, plant residues and food waste (Čičková et al., 2015). Frass (the rearing process by-product), which is essentially a mixture of larval excrement, undigested organic waste and shed exoskeletons, has the potential to be used as a soil

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improver or organic fertiliser (Kebli and Sinaj, 2017).

While the nutrient content of frass depends on the insects' diet, it can generally be described as having high organic matter and available nutrient content including nitrogen (N), phosphorus (P) and potassium (K), and a narrow ratio of carbon (C) to N. Furthermore, it potentially contains microbes which promote plant growth by the release of plant hormones or suppression of pathogens (Poveda et al., 2019) if the frass has not been sterilised, for example through thermal treatment (Gold et al., 2018). On the other hand, Kawasaki et al. (2020) identified in non-sterilised black solider fly frass a high relative abundance of Xanthomonadaceae, a bacterial family containing a plant-pathogenic genus. Frass has been demonstrated to be an effective fertiliser in field-grown maize (Quilliam et al., 2020) and cabbage (Choi et al., 2009) and in pot trials monitoring growth of chard (Poveda et al., 2019), ryegrass and lettuce (Kebli and Sinaj, 2017).

The profound effect of larval feedstock makes generalisations about frass nutrient content difficult. However, Poveda et al. (2019) tested three contrasting mealworm diets and observed the most proteinaceous regime to generate frass with the highest calcium (Ca), molybdenum (Mo) and sulphur (S) concentrations. The same authors reported diets relatively enriched in carbohydrates led to frass with higher P, K, magnesium (Mg), manganese (Mn) and iron (Fe) contents. Frass addition and breakdown can increase the soil content of C and N (Eo et al., 2017). Both elements are released by degradation of the main exoskeleton component chitin (Jacquiod et al., 2013). Undigested dietary amino acids and proteins (Fielding et al., 2013) as well as nitrogen metabolism compounds such as uric acid and allantoin (Kagata and Ohgushi, 2011) can also be sources of C and N. Until now, no comparison of immediately and potentially plant-available nutrient contents of the frass of commercially important insect larvae has been made.

The input of available C to soil from frass is likely to induce microbial growth which can sequester a pulse of released N (Lovett and Ruesink, 1995), with the microbial necromass later becoming a source of N (Fielding et al., 2013) and other nutrients. The increased amount of C, N and other nutrients in soil following frass addition may also stimulate microbial breakdown of native organic matter (Behie and Bidochka, 2013), commonly termed the priming effect (Kuzyakov et al., 2000). The chitin component of frass has been observed to markedly effect growth in soil populations of chitinolytic fungi (such as *Aspergillus*) and bacteria (Sarathchandra et al., 1996). Identification of bacterial classes responsible for this is hindered by the difficulty in distinguishing genuine degraders and "cheaters" utilising chitin breakdown products (Jacquiod et al., 2013). The stimulation of chitinolytic bacteria can possibly lead to suppression of plant pathogens and some plant diseases caused by nematodes or fungi (Kielak et al., 2013). Poveda et al. (2019) identified in frass genera of plant growth promoting rhizobacteria that are capable of providing auxins, gibberellins, siderophores and protection against pathogens. Respective examples of these include *Pseudomonas*, *Acinetobacter*, *Pantoea* and *Brevibacillus*. It should not be overlooked that larvae can also utilise fungi and bacteria as food. Some fungi may survive passing through the digestive tract while some insect gut bacteria are transferred to the frass, the microbial composition of which is greatly affected by the feedstock (Gold et al., 2018). It is unknown whether the amendment of soil by different frass types consistently stimulates microbial nitrification, respiration and growth, nor whether the relative proportions of bacteria, archaea and fungi are impacted.

Heavy metals can negatively affect microorganisms in various ways, for instance inducing enzymatic dysfunction or causing membrane and DNA damage (Bruins et al., 2000). Microbial defences against heavy metals are exemplified by the efflux pumps utilised by bacteria against excess Cu or Cd. Such systems have a high maintenance energy demand, causing reduced efficiency of substrate utilisation and microbial biomass (Giller et al., 2009). Heavy metal inputs to soils may come from natural atmospheric deposition or anthropogenic inputs such as application of sludges and phosphate fertilisers to agricultural land. High application rates and frequent use of these can effect heavy metal accumulation as they may contain zinc (Zn), cadmium (Cd), nickel (Ni) and copper (Cu) as impurities (Wuana and Okieimen, 2011; Alloway, 2013). Furthermore, manures can represent a considerable input to soils of Zn (Alloway, 2013) and Cu. Cu can also be potentially elevated by excessive fungicide application (Oorts, 2013). Biochars have been produced from mealworm frass and utilised as adsorbents of heavy metals from solutions (Yang et al., 2019). To our knowledge no investigations have assessed whether non-pyrolysed frass, which is carbonaceous and potentially adsorptive, can reduce heavy metal bioavailability and thereby their microbial impacts in soils.

The aims of this investigation were to ascertain whether:

- Frass types differ in terms of their extractable macro- and micronutrients
- Frass application to soil stimulates microbial carbon and nitrogen mineralisation
- Soil amendment by frass induces microbial growth and changes at the domain/kingdom level
- Frass is suitable as an amendment where the goal is to reduce heavy metal bioavailability and, if so, the ameliorative mechanisms can be deduced

2. Materials and methods

2.1. Frass, vermicompost and soil characterisation

2.1.1. Background

Frass of black soldier fly (BSFF), buffalo worm (BWF) and mealworm (MWF) was provided by Vivara Natuurbeschermingsproducten, Vierlingsbeek, Netherlands. Mealworm and buffalo worm larvae were reared on Insectus Mealworm Grow (Mijten nv, Bekkevoort, Belgium) which contains 20% protein, 4% fat, 1.01% Ca, 0.76% P and 0.23% Mg, with carrots supplementing the feed. Black soldier flies were fed with a residual product from the wheat processing industry composed mainly of wheat bran. Impacts of frass on soil chemistry, microbial processes and abundances of domains/kingdoms were contrasted with that of a commercially available vermicompost, which was obtained from Wurmwelten, Dassel, Germany.

For the frass amendment incubation experiment, soil was collected from the top 30 cm of a pasture field in Bedburg-Hau, Germany, sieved (<2 mm) and stored at 5 °C for one month prior to the experiment. The grassland soil has the following general properties: pH 5.35 (in 0.01 M $CaCl_2$), sandy loam textural class (70% sand, 17% silt, 13% clay), 2.38% organic matter, 1.1% C and 0.12% N.

For the metal toxicity mitigation experiment, a carbon-poor substrate was made, comprising a 1:1 mixture of quartz sand (0.1–0.5 mm, RKW, Falkenstein, Germany) and air-dried (sieved <4 mm) soil. The soil used was the B horizon of a stagnic luvisol used as grassland (textural class silt loam with 10% sand, 80% silt and 10% clay, pH 5.48 in 0.01 M $CaCl_2$, 0.29% TOC, <0.05% N) from Neulouisendorf, Germany.

2.1.2. Analyses

All soil amendments were air dried for 3 days and sieved (<1 mm) prior to soil application or analyses, which comprised organic matter, total N, C/N ratio (assuming organic matter to be 58% C), pH in deionised water (amendment: water ratio 1:10), electrical conductivity (amendment: water ratio 1:10), extractable nutrients (NH_4, NO_3, Mg, P, K, B, Cu, Fe, Mn, Zn) in 0.01 M $CaCl_2$ and extractable nutrients (Ca, Mg, P, K, B, Cu, Fe, Mn, Zn) in Mehlich 3 (Ziadi and Tran, 2008). Elemental nutrient concentrations were determined via an Optima 8000 inductively coupled plasma optical emission spectrometer (ICP-OES) (PerkinElmer, Baesweiler, Germany). Nitrate and NH_4 were measured using an AA3 HR Nutrient Autoanalyzer (SEAL Analytical GmbH, Norderstedt, Germany).

2.2. Frass amendment incubation experiment

2.2.1. Design

The incubation experiment was carried out for 28 days at 22 °C in 1 L glass jars. The samples contained 100 g dry weight soil adjusted to 50% of the water-holding capacity plus various concentrations of soil amendment. The following treatments were carried out in replicates of seven:

(1) Control (no amendment)
(2) Soil amended with 2.5% (w/w) vermicompost (Vermi 2.5)
(3) Soil amended with 5% (w/w) vermicompost (Vermi 5)
(4) Soil amended with 2.5% (w/w) black soldier fly frass (BSFF 2.5)
(5) Soil amended with 5% (w/w) black soldier fly frass (BSFF 5)
(6) Soil amended with 2.5% (w/w) buffalo worm frass (BWF 2.5)
(7) Soil amended with 5% (w/w) buffalo worm frass (BWF 5)
(8) Soil amended with 2.5% (w/w) mealworm frass (MWF 2.5)
(9) Soil amended with 5% (w/w) mealworm frass (MWF 5)

2.2.2. Analyses

Evolved CO_2 during the 28-day incubation was absorbed in alkali traps containing NaOH solution; depending on the treatment and stage of incubation, the molarity of these traps and their corresponding blanks ranged from 0.25 to 7.0 M. A trap was removed from each jar after 3, 7, 14, 21 and 28 days and replaced with a new one. One mL aliquots of each removed trap were back titrated with 0.1 M HCl (after addition of 20 mL deionised water and 5 mL 1.0 M $BaCl_2$ solution) using a Titroline® 6000 automatic titrator (SI Analytics, Mainz, Germany). Quantities of evolved CO_2 were used to calculate cumulated respired CO_2-C.

At the end of the incubation period, 15 g subsamples of soil were taken from each vessel for determinations of extractable ammonium, nitrite and nitrate, ergosterol and DNA (as a proxy for soil microbial biomass) concentrations (μg g $soil^{-1}$). For calculating the microbial quotient (microbial C expressed as a percentage of total C), DNA concentrations were converted to microbial C by multiplying by a factor of 6 (Joergensen and Emmerling, 2006). The microbial biomass specific respiration rate (CO_2-C evolved per unit of microbial biomass C per day) was calculated using the respiration data of the last seven days of the incubation. Ten g of moist soil were extracted for 30 min by oscillating shaking at 200 rev min^{-1} with 40 mL 0.5 M K_2SO_4 and analysed for ammonium, nitrite and nitrate in an AA3 HR Nutrient Autoanalyzer (SEAL Analytical GmbH, Norderstedt, Germany).

Total genomic DNA was extracted from 0.5 g of fresh soil using a FastDNA™ SPIN Kit for Soil and FastPrep®-24 bead-based homogenizer (both MP Bio, Santa Ana, CA) according to the manufacturer's instructions as modified by Hemkemeyer et al. (2014). The concentration of extracted DNA was measured based on the intercalating dye QuantiFluor® dsDNA System (Promega GmbH, Mannheim, Germany) in a FLUOstar® Omega microplate reader (BMG Labtech, Ortenberg, Germany) at 485 nm excitation and 520 nm emission. Quantitative PCR of archaeal and bacterial 16S rRNA genes was conducted in a LightCycler® 480 II using LightCycler® 480 Probes Master (Roche, Penzberg, Germany). PCR reactions were carried out in a 20 μL total volume which contained 10 μL master mix, 0.5 μM of each primer (Table 1), 0.2 μM probe (Table 1) and 2 μL of template DNA. In order to check for inhibitory effects, reactions of each sample were run in duplicate, with one half being supplied with 10-fold and the other half with 50-fold dilutions of template DNA. Reaction conditions started with 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s, 60 °C for 10 s, and 72 °C for 15 s. DNA fragments of *Methanobacterium oryzae* and *Bacillus subtilis* cloned into pGEM®-T vector (Promega) served as standards ranging from 10^2 to 10^7 and 10^4–10^8 copies μL^{-1} template, respectively. Data were pre-processed using "Abs Quant/2nd Derivative Max"-analysis of the instrument accompanying software (1.5.0 SP4) and obtained crossing threshold (CT) values were further processed in Microsoft Excel 2010.

Table 1
Primers and probes (Yu et al., 2005) used in the present study.

Taxon	Primers & probes	Sequence (5′-3′)
Archaea	ARC787F	ATTAGATACCCSBGTAGTCC
	ARC1059R	GCCATGCACCWCCTCT
	ARC915F	HEX-AGGAATTGGCGG GGGAGCAC-BHQ1
Bacteria	BAC338F	ACTCCTACGGGAGGCAG
	BAC805R	GACTACCAGGGTATCTAATCC
	BAC516F	FAM-TGCCAGCAGCCGCGGTAATAC-BHQ1

Fungi were quantified by measuring the ergosterol content since melting curve analysis of a SYBR Green-based qPCR indicated that fungal ITS1 sequences differed too strongly between the soil treatments, prohibiting their quantitative comparison. Ergosterol content (an indicator of saprotrophic fungi) was measured according to Djajakirana et al. (1996). Briefly, 2 g moist soil was extracted with 100 mL ethanol for 30 min by oscillating shaking at 250 rev min^{-1} and then filtered (Whatman GF/A, 1.6 μm). Ergosterol determination was carried out via reversed-phase HPLC analysis at 26 °C, using a 125 mm × 4 mm Sphereclone 5 μm ODS II column with a Phenomenex guard column (4 mm × 3 mm). Chromatography was performed isocratically with methanol (100%) and a resolution of detection of 282 nm (Dionex UVD 170 S). Fungal proportion of microbial biomass was calculated based on the assumptions that DNA is 37.7% C and ergosterol is composed of 84.8% C.

2.3. Heavy metal toxicity mitigation experiment

2.3.1. Design

The incubation experiment was carried out for 56 days at 22 °C with all samples (100 g dry weight soil) adjusted to 50% of the maximum water-holding capacity in 1 L glass jars after 3% (w/w) application of a soil amendment. Samples were amended with 20 mg kg^{-1} Cd, 140 mg kg^{-1} Ni (both in line with the German threshold for land intended for residential use; European Commission, 2007), 140 mg kg^{-1} Cu and 400 mg kg^{-1} Zn (both selected to be markedly higher than German threshold values for agricultural land; AbfKlärV, 1992), applied in the form of dissolved sulphate salts. Nutrients were simultaneously applied in this solution at a rate of 100 mg kg^{-1} K, 40 mg kg^{-1} Ca, 20 mg kg^{1} Mg, 30 mg kg^{-1} NH_4-N, NO_3-N and P, 0.1 mg kg^{-1} B, 0.3 mg kg^{-1} Mo, 2.5 mg kg^{-1} Fe and 0.8 mg kg^{-1} Mn. A further solution, containing the same quantities of nutrients but no heavy metals, was added after 28 days to each vessel, bringing the water-holding capacity to 60%.

All of the following treatments were carried out in replicates of seven:

(1) Control (only heavy metals applied)
(2) Substrate amended with heavy metals and 3% (w/w) vermicompost (Vermi)
(3) Substrate amended with heavy metals and 3% (w/w) black soldier fly frass (BSFF)
(4) Substrate amended with heavy metals and 3% (w/w) buffalo worm frass (BWF)
(5) Substrate amended with heavy metals and 3% (w/w) mealworm frass (MWF)

2.3.2. Analyses

At the end of the incubation, samples from each vessel were taken for microbial biomass C determination by chloroform fumigation extraction including a pre-extraction step (Vance et al., 1987; Mueller et al., 1992). Briefly, 50 g of sample from each vessel was pre-extracted by horizontal shaking for 30 min with 200 mL 0.05 M K_2SO_4 at 200 rev min^{-1}. The pre-extracts were frozen for later analysis; of the pre-extracted soil, fumigated and non-fumigated 10 g portions were extracted for 30 min by horizontal shaking at 200 rev min^{-1} with 40 mL 0.5 M K_2SO_4.

Fumigation was carried out in desiccators for 24 h at room temperature. Organic C and N in the extracts were measured using a multi N/C 2100S analyser (Analytik Jena AG, Jena, Germany). Microbial biomass C was calculated as E_C / k_{EC}, where E_C = (organic C extracted from fumigated soils) − (organic C extracted from non-fumigated soils) and k_{EC}, the correction factor for MBC = 0.45 (Wu et al., 1990). Samples from each vessel were also used to determine 0.01 M $CaCl_2$-extractable Cd, Cu, Ni and Zn via ICP-OES.

2.4. Statistical analyses

Data presented in the tables and graphs are arithmetic means and refer to oven-dry (24 h, 105 °C) soil. All statistical calculations were performed using the packages agricolae, MASS, tidyverse, readxl and latex2exp in the computing environment R (R Core Team, 2019). Differences in means were tested for statistical significance by using one-way ANOVA. In order to check whether conditions for ANOVA were fulfilled, residuals were plotted against fitted values to assess the homoscedasticity. Normal distribution was checked visually by a Normal Quantile-Quantile plot. When presumptions were not fulfilled Box & Cox power transformations were carried out. Where a significant difference ($p < 0.05$) was observed between treatments, a post hoc Tukey HSD-test was conducted.

3. Results

3.1. Soil amendment characterisation

Although obviously no comparison was possible for Ca or the Mehlich 3 components NO_3 and NH_4, extractable macro- and micronutrient concentrations were consistently higher in the more aggressive Mehlich 3 solution than in 0.01 M $CaCl_2$ (Tables 2 and 3). The contrast was particularly marked for Mg (Table 2) and Fe, Mn and Zn (Table 3).

Table 2
Mean values of macronutrients (g kg^{-1}) in vermicompost (Vermi), black soldier fly frass (BSFF), buffalo worm frass (BWF) and mealworm frass (MWF) extracted by 0.01 M $CaCl_2$ or Mehlich 3. Values show arithmetic means and are followed by standard errors of the mean in brackets (n = 7). For each extractant, different letters within a column indicate significant differences ($p < 0.05$).

Amendment and extractant	NO_3	NH_4	Ca	P	K	Mg
0.01 M $CaCl_2$						
Vermi	5.70 (0.19) c	0.04 (0.002) a	–	0.37 (0.004) a	1.77 (0.04) a	0.92 (0.01) a
BSFF	0.54 (0.01) a	7.96 (0.40) c	–	14.41 (0.50) d	22.36 (0.62) c	2.78 (0.03) d
BWF	0.60 (0.02) b	1.85 (0.04) b	–	1.63 (0.04) b	7.56 (0.71) b	1.70 (0.01) c
MWF	0.64 (0.01) b	1.68 (0.07) b	–	2.65 (0.03) c	10.07 (1.14) b	1.56 (0.007) b
Mehlich 3						
Vermi	–	–	9.64 (0.18) c	0.64 (0.02) a	1.81 (0.03) a	1.28 (0.02) a
BSFF	–	–	0.47 (0.006) a	19.77 (0.40) d	24.03 (0.21) d	7.09 (0.17) c
BWF	–	–	4.76 (0.12) b	7.77 (0.18) b	16.21 (0.24) b	4.49 (0.11) b
MWF	–	–	4.35 (0.18) b	9.77 (0.35) b	22.40 (0.72) c	4.89 (0.18) b

Table 3
Mean values of micronutrients (mg kg^{-1}) in vermicompost (Vermi), black soldier fly frass (BSFF), buffalo worm frass (BWF) and mealworm frass (MWF) extracted by 0.01 M $CaCl_2$ or Mehlich 3. Values show arithmetic means and are followed by standard errors of the mean in brackets (n = 7). For each extractant, different letters within a column indicate significant differences ($p < 0.05$). "nd" denotes values below the limit of detection.

Amendment and extractant	B	Cu	Fe	Mn	Zn
0.01 M $CaCl_2$					
Vermi	0.69 (0.41) a	0.10 (0.001) a	nd	2.62 (0.02) a	0.21 (0.01) a
BSFF	8.09 (1.17) b	8.97 (0.08) b	14.88 (0.32) a	19.42 (0.15) d	14.99 (0.18) d
BWF	9.21 (0.56) bc	13.64 (0.88) c	17.65 (0.24) c	9.56 (0.05) c	11.87 (0.34) c
MWF	10.99 (0.37) c	14.57 (1.22) c	16.45 (0.18) b	6.01 (0.05) b	7.75 (0.29) b
Mehlich 3					
Vermi	0.77 (0.26) a	2.71 (0.03) a	187 (2.41) a	22.56 (0.30) a	36.04 (0.49) a
BSFF	7.78 (0.41) b	17.18 (0.64) b	183 (5.62) a	152 (5.29) bc	284 (8.63) c
BWF	11.00 (0.29) c	24.62 (6.45) b	179 (5.20) a	140 (4.90) b	136 (4.53) b
MWF	13.94 (0.56) d	25.03 (2.79) b	192 (5.55) a	163 (5.46) c	154 (4.93) b

Vermicompost had higher extractable NO_3 and Ca concentrations than frass whereas frass featured higher NH_4 concentrations than vermicompost (Table 2). The three frass types had markedly higher extractable concentrations of all other nutrients (Tables 2 and 3) than vermicompost did. BSFF was significantly richer in extractable NH_4, P, K, Mg (Table 2), Zn and 0.01 M $CaCl_2$-extractable Mn (Table 3) but clearly poorer in extractable Ca (Table 2), B and 0.01 M $CaCl_2$-extractable Cu and Fe (Table 3) than BWF and MWF. Overall, frass was richer in organic matter and N than vermicompost, with a narrower C:N ratio and a higher pH and electrical conductivity (Table 4). BWF and MWF contained respectively 1.49 and 1.15% more total N than BSFF.

3.2. Frass amendment incubation experiment

All amendments stimulated greater soil microbial respiration than in the unamended control (Table 5). The 5% frass application rate caused significantly greater respiratory response than the 2.5% frass application rate. This was not the case with the 2.5% and 5% application rates of vermicompost. Frass-induced microbial respiration was markedly greater than that induced by vermicompost. Both application rates of BWF and MWF caused significantly greater respiration than in the corresponding BSFF regimes. However, the highest respiratory response of the 5% frass-amended treatments was only matched with significantly increased microbial biomass (expressed in extractable DNA

Table 4
Mean values of organic matter (%), total N (%), C/N ratio (all n = 3), pH and electrical conductivity (both n = 7) in vermicompost (Vermi), black soldier fly frass (BSFF), buffalo worm frass (BWF) and mealworm frass (MWF). Values show arithmetic means and are followed in brackets by standard errors of the mean.

Amendment	Organic matter, %	Total N, %	C/N ratio	pH	Electrical conductivity (S/m)
Vermi	50.47 (1.32) a	1.27 (0.01) a	23.12 (0.80) c	6.07 (0.01) a	0.19 (0.01) a
BSFF	77.10 (0.17) bc	2.80 (0.04) b	16.00 (0.23) b	6.78 (0.01) d	0.48 (0.01) d
BWF	78.87 (0.20) c	4.29 (0.02) d	10.67 (0.07) a	6.25 (0.01) b	0.32 (0.01) b
MWF	75.50 (0.06) b	3.95 (0.02) c	11.08 (0.06) a	6.61 (0.01) c	0.43 (0.01) c

Table 5
Mean values of the cumulated respiration (mg CO_2-C/g soil/28 days), extractable DNA (mg kg^{-1}), microbial quotient (C_{mic}/C_{org} expressed as a percentage) and the microbial biomass specific respiration rate (respired CO_2-C, mg per day/C_{mic} in g). Soil was amended with 2.5 or 5% (w/w) vermicompost (Vermi), black soldier fly frass (BSFF), buffalo worm frass (BWF) or mealworm frass (MWF). Values show arithmetic means and are followed by standard errors of the mean in brackets (n = 7). Within a column, different letters indicate significant differences.

Treatment	Cumulated Respiration	Extractable DNA (mg kg^{-1})	Microbial quotient (%)	Microbial biomass specific respiration rate (mg CO_2-C d^{-1} g^{-1} C_{mic})
Control	0.22 (0.01) a	6.08 (0.38) a	0.33 (0.02) a	4.99 (1.53) a
Vermi 2.5	1.45 (0.13) b	13.48 (0.84) b	0.44 (0.03) b	21.08 (4.98) ab
Vermi 5	1.62 (0.13) b	22.64 (1.12) c	0.53 (0.03) bc	14.41 (1.40) ab
BSFF 2.5	9.95 (0.08) c	45.78 (3.96) d	1.24 (0.11) e	20.30 (4.92) ab
BSFF 5	14.95 (0.96) d	63.91 (3.72) e	1.15 (0.07) e	41.23 (7.06) abc
BWF 2.5	14.38 (0.26) d	40.57 (2.81) d	1.09 (0.08) e	47.74 (9.04) bc
BWF 5	22.94 (1.71) e	39.88 (4.04) d	0.71 (0.07) cd	98.93 (20.85) d
MWF 2.5	12.95 (0.09) d	41.20 (2.26) d	1.13 (0.06) e	32.36 (3.56) abc
MWF 5	20.72 (0.76) e	42.54 (1.67) d	0.78 (0.03) d	70.88 (10.79) cd

concentrations) in the BSFF-amended replicates (Table 5). Indeed, the 5% application rates of BWF and MWF had significantly lower microbial quotients than the 2.5% rates which was not the case for BSFF (Table 5). The microbial biomass-specific respiration rates were higher in the 5% frass-amended regimes, significantly so for BWF (Table 5).

Relative to the control, all soil amendments stimulated bacterial and archaeal communities. The 5% application rate caused significantly higher 16S rRNA gene copy numbers of bacteria where BSFF or MWF was applied, particularly for BSFF (Fig. 1). The converse was the case with regard to archaea: the 2.5% frass application rate induced higher 16S rRNA gene copy numbers than did 5%, significantly so for MWF (Fig. 1).

The extractable ergosterol concentrations revealed fungal biomass to have been stimulated by all soil amendments. This stimulation was disproportionately high at the 5% application rate of frass, where doubling the frass application rate induced an approximately four-fold fungal biomass increase (Fig. 2). Fig. 3 expresses the ergosterol C as a percentage of DNA carbon. The 2.5% frass application rate did not induce any significant increases relative to the control, whereas the 5% application rate caused a significant increase in the proportion of microbial biomass that was fungal.

The 5% frass application rate led to significantly higher concentrations of extractable NH_4 at the end of the incubation (Table 6). Overall, the application of BWF and MWF to soil led to a substantially greater release of inorganic N than BSFF (Table 6). A significant proportion of the NH_4 applied to the soil via frass application has been nitrified. This is evidenced by the higher extractable NO_3 concentrations than in the control, significantly so in the 2.5% MWF-amended samples (Table 6). Interestingly, very high nitrite concentrations were observed in the samples amended with 2.5% BWF and MWF (Table 6).

3.3. Heavy metal toxicity mitigation experiment

Microbial biomass C extracted from the heavy-metal contaminated substrate after 56 days' incubation was significantly higher where BSFF or BWF was applied than in the non-amended control (Fig. 4). Vermicompost and MWF did not induce significant increases in microbial biomass relative to the control. Nevertheless, their application significantly reduced the concentrations of 0.01 M $CaCl_2$-extractable Cu relative to the control (Fig. 5). The 0.01 M $CaCl_2$-extractable concentrations of Ni, Cd and Zn in the control treatment were respectively 46, 51 and 58% of that applied. This indicates the relative bioavailability of these metals in contrast to Cu, whose 0.01 M $CaCl_2$-extractable concentration was only 5%. Frass application caused significant reductions of all extractable metals relative to the control, with the exception of Ni in the BSFF-amended replicates (Fig. 5).

4. Discussion

4.1. Frass amendment incubation experiment

The frass used in our experiments contained between 2.8 and 4.3% N (Table 4). This is comparable with the value of 3.1% documented by

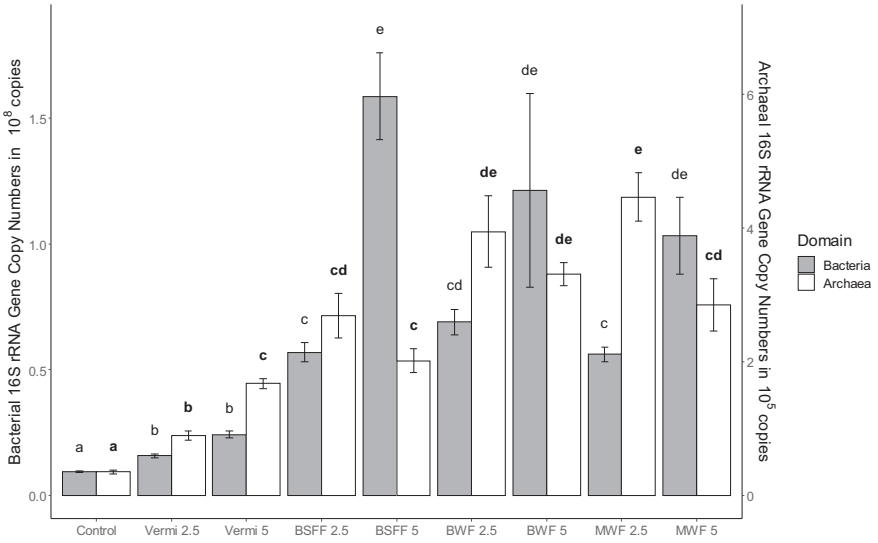

Fig. 1. Mean 16S rRNA gene copy numbers of bacteria (grey bars, standard letters) and archaea (white bars, bold letters) per gram of soil (n = 7). Soil was amended with 2.5 or 5% (w/w) vermicompost (Vermi), black soldier fly frass (BSFF), buffalo worm frass (BWF) or mealworm frass (MWF). For each microbial domain, different letters indicate significant differences between the means of the treatments ($p < 0.05$). Error bars display the standard error.

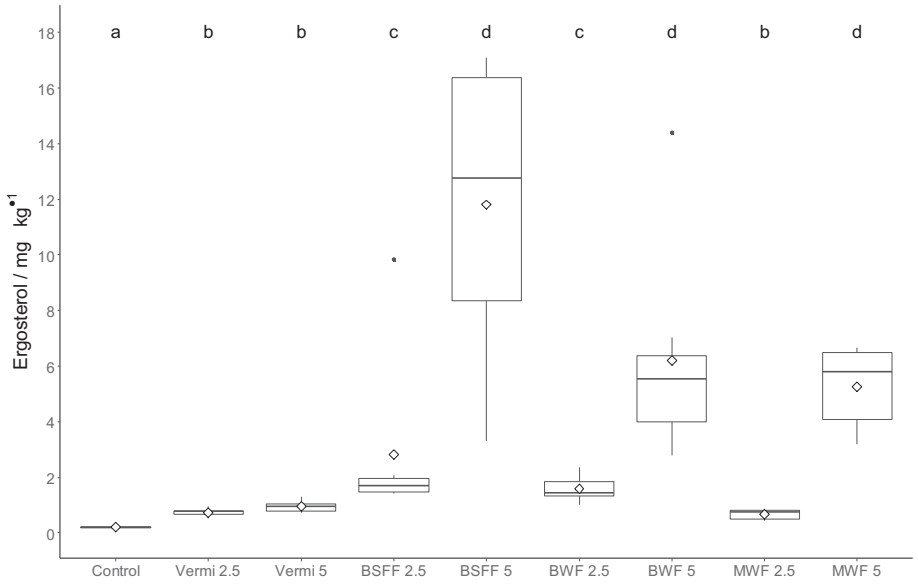

Fig. 2. Mean values of ergosterol (mg kg^{-1}) extracted from soil after 28 days' incubation (n = 7). Soil was amended with 2.5 or 5% (w/w) vermicompost (Vermi), black soldier fly frass (BSFF), buffalo worm frass (BWF) or mealworm frass (MWF). Different letters indicate significant differences between the means (denoted by diamonds) of the treatments ($p < 0.05$).

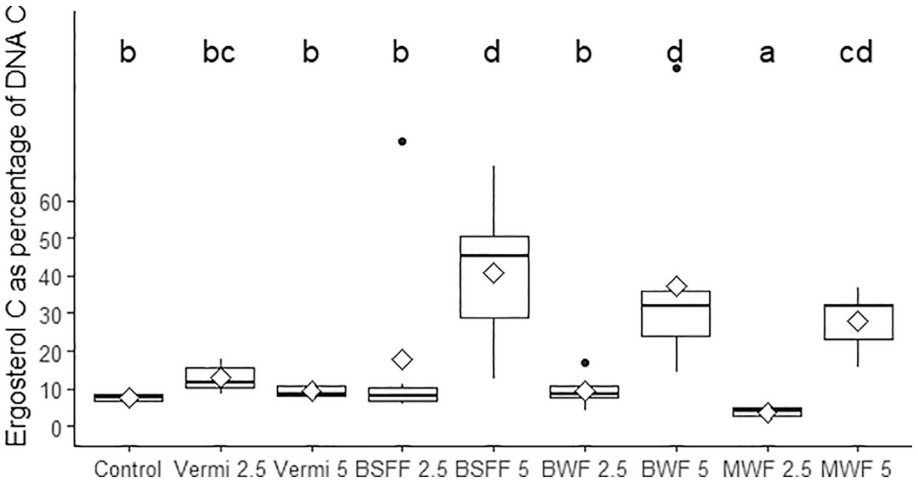

Fig. 3. Mean values of ergosterol carbon (extracted from soil after 28 days' incubation) expressed as a percentage of DNA carbon (n = 7), based on assumptions that ergosterol is composed of 84.8% C and DNA 37.7% C. Soil was amended with 2.5 or 5% (w/w) vermicompost (Vermi), black soldier fly frass (BSFF), buffalo worm frass (BWF) or mealworm frass (MWF). Different letters indicate significant differences between the means (denoted by diamonds) of the treatments ($p < 0.05$).

Frost and Hunter (2004), although theirs was an ecosystem study that did not focus on frass of any particular insect larvae. The BSFF used in this study contained 77% organic matter (Table 4). Kebli and Sinaj (2017) recorded a similar value of 76%. These authors reported 0.01 M $CaCl_2$-extractable B, Zn and Cu values in BSFF of 0.03, 7.2 and 3.6 mg kg^{-1}, respectively, which are markedly lower than ours, particularly for B (Table 3). They also observed Olsen-extractable P to be 4.3 g kg^{-1} in BSFF, which was lower than the 0.01 M $CaCl_2$-extractable P value we recorded of 14.4 g kg^{-1} (Table 2). Kawasaki et al. (2020) noted a C/N ratio of 16.6 in BSFF produced from larvae reared on household waste, similar to the value we observed (Table 4). These authors also measured comparable concentrations of extractable NH_4, NO_3 and Mn (respectively 11.3 g kg^{-1}, 0.44 g kg^{-1} and 100 mg kg^{-1}). However, concentrations of extractable P, K and Mg (respectively 0.5, 0.7 and 0.9 g kg^{-1}) were considerably lower than in our study.

These discrepancies exemplify the difficulty in generalising about the nutrient content of frass as it largely depends on the larval feedstock and processing. Nevertheless, the work of Poveda et al. (2019) highlighted that carbohydrate-rich diets yield frass relatively rich in P, K, Mg and Mn, whereas protein-rich diets lead to frass with higher Ca concentrations. Our results support this: the frass used in our experiment came from mealworms and buffalo worms reared on relatively protein-rich feedstocks whereas the black soldier flies were provided with carbohydrate-rich feed. Frass has been described as rich in available carbon (Kagata and Ohgushi, 2012). The organic matter content of the three frass types we used was similar (Table 4) but we can only speculate as to whether the amount of labile C contained in each frass type was comparable. However, BSFF compared to MWF and BWF featured remarkably higher levels of extractable NH_4, P, K, Mg (Table 2), Mn and Zn (Table 3), all of which participate in myriad cellular processes and enzymatic reactions. This may be the reason why microbial biomass, as indicated by extractable DNA concentrations, was significantly greatest with the 5% frass application rate only when BSFF was applied (Table 5).

Table 6
Mean values of extractable ammonium, nitrite, nitrate and total inorganic N (mg kg^{-1}) from soil after 28 days' incubation. Soil was amended with 2.5 or 5% (w/w) vermicompost (Vermi), black soldier fly frass (BSFF), buffalo worm frass (BWF) or mealworm frass (MWF). Values show arithmetic means and are followed by standard errors of the mean in brackets (n = 7 except where indicated). Different letters within a column indicate significant differences. "nd" denotes values below the limit of detection.

Treatment	NH_4-N, mg kg^{-1}	NO_2-N, mg kg^{-1}	NO_3-N, mg kg^{-1}	Total inorganic N
Control	2.67 (0.15) a	nd	29.60 (2.33) a	32.27 (2.45)
Vermi 2.5	2.01 (0.10) a	0.55[b] (0.39)	79.89 (4.35) ab	82.45 (4.52)
Vermi 5	2.54 (0.19) a	0.32[a]	105.81 (9.61) ab	108.67 (9.64)
BSFF 2.5	3.95 (0.28) a	nd	140.59 (10.03) ab	144.54 (10.02)
BSFF 5	307.71 (63.17) b	nd	117.78 (24.61) ab	425.49 (42.89)
BWF 2.5	520.28 (49.20) c	129.73 (21.07)	24.81 (7.69) a	674.82 (48.52)
BWF 5	967.02 (30.54) d	nd	67.36 (1.08) ab	1034.38 (30.15)
MWF 2.5	290.68 (74.91) b	139.44 (44.56)	201.80 (95.42) b	631.92 (71.20)
MWF 5	857.60 (24.88) d	nd	139.31 (3.86) ab	996.91 (28.51)

[a] n = 1.
[b] n = 2.

Considerable C mineralisation was induced by frass application (Table 5). The C mineralised, when considering the native soil C and that added with the amendment, was equivalent to 2% in the control treatment and 7% where vermicompost was applied. The substantial amounts of labile C in the frass become apparent when mineralised C following their application is calculated: respectively 66, 61 and 45% for BWF, MWF and BSFF. After grasshopper frass was incubated in soil for the same time as in our experiment, Fielding et al. (2013) reported similar C mineralisation to that of BSFF in the present study. While the microbial cumulated respiratory response to MWF and BWF was greater than the response to BSFF amendment at both application rates, there was no corresponding microbial biomass increase at the 5% application rate of MWF or BWF (Table 5). This is reflected in the inversely proportional microbial quotient and microbial biomass specific respiration rates. The former significantly fell at the 5% application rates of MWF and BWF, whereas the latter increased, significantly for BWF (Table 5). Higher microbial quotients point to greater availability of organic matter to soil microbes. Higher microbial biomass specific respiration rates on the other hand indicate either a relatively young, inefficient microbial biomass, or one that is utilising more carbon catabolically, at the expense of that invested in biomass, due to stress (Joergensen and Emmerling, 2006). Given that the extractable DNA did not increase at the 5% application rates of MWF and BWF (Table 5), a young inefficient biomass can be discounted. What has caused the stress, expressed in the higher microbial biomass specific respiration rates, is not obvious. These frass types do not have a higher salinity than BSFF (Table 4) but possibly the extractable concentrations of Ca being ten times higher than in the BSFF (Table 2) with significantly lower concentrations of extractable NH_4, P, K, Mg (Table 2), Mn and Zn (Table 3) constitute a nutrient imbalance that hindered microbial biomass formation.

Another possible explanation of this is that biomass growth in the 5% MWF and BWF treatments of our relatively short-term experiment became C-limited. C mineralisation was greatest in the first week of incubation (data not shown). We could assume the higher N contents of MWF and BWF (Table 4) provided an excess of available N. Accordingly, it is possible the stimulatory effect of this N addition was dependent on release of C from more recalcitrant components of MWF and BWF after depletion of soluble C and was therefore not evident in our results.

A perusal of the extractable inorganic N after the incubation (Table 6) allows the conclusion that, relative to the control, all treatments led to net N mineralisation. Frost and Hunter (2004) also observed increased N (particularly NH_4) in frass amended soils. Atiyeh et al. (2000) reported N release from vermicompost to result in higher NO_3 concentrations, as was the case in our experiment. Even though BSFF had more easily extractable NH_4 than the other frass types (Table 2), BWF and MWF contained higher levels of total N (Table 4). The biodegradation of MWF and BWF must release more formerly organically bound N that caused the substantially higher release of inorganic N, some of which can be oxidised to nitrite (Table 6). This could shed light on the contrasting microbial responses to the three frass types. Application of 2.5% MWF and BWF induced a marked spike in extractable nitrite concentrations (Table 6) and showed the highest archaeal 16S rRNA gene copy numbers in contrast to the 5% application rate (Fig. 1). These observations may be associated, given that a diverse range of archaea comprise the most numerous soil nitrifiers of NH_4 to nitrite (Francis et al., 2007). Furthermore, nitrite may build up to a certain extent after N fertilisation as the rates of NH_4 and nitrite

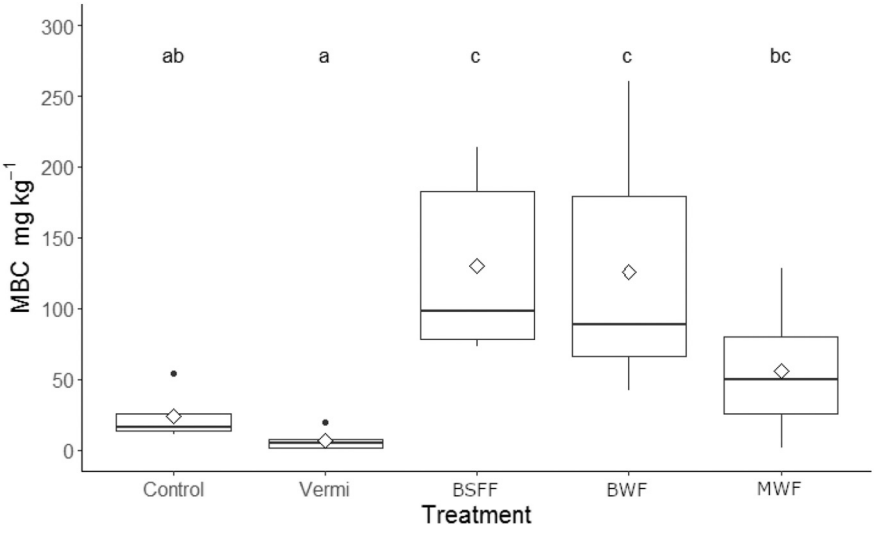

Fig. 4. Mean values of microbial biomass carbon (MBC, mg kg^{-1}) extracted from heavy-metal spiked soils after 56 days' incubation (n = 7). Soil was amended with 3% (w/w) vermicompost (Vermi), black soldier fly frass (BSFF), buffalo worm frass (BWF) or mealworm frass (MWF). Diamonds show arithmetic means. Different letters indicate significant differences between the means of the treatments ($p < 0.05$).

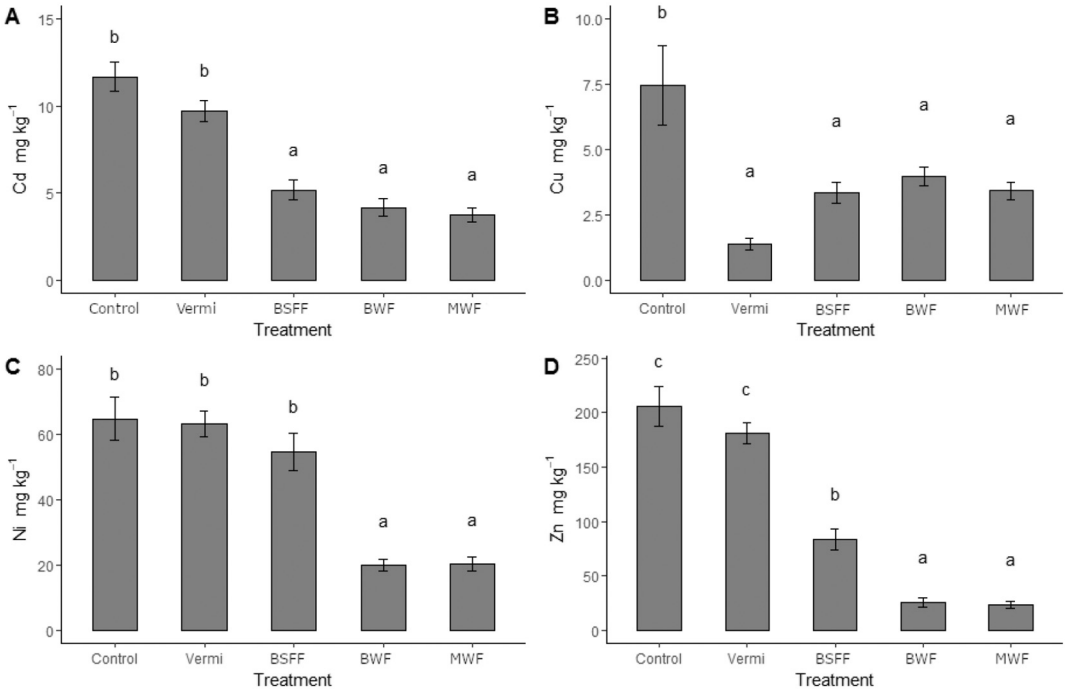

Fig. 5. Mean values of 0.01 M $CaCl_2$-extractable Cd, Cu, Ni and Zn (mg kg^{-1}) from amended soils after 56 days' incubation (n = 7). Soil was amended with 3% (w/w) vermicompost (Vermi), black soldier fly frass (BSFF), buffalo worm frass (BWF) or mealworm frass (MWF). Different letters indicate significant differences between the means of the treatments ($p < 0.05$). Error bars display the standard error of the mean.

oxidation are not linked (Taylor et al., 2019). As the oxidation rate of NH_4 can exceed that of nitrite due to it representing a better energy source, it is probable that the build-up of both nitrite and archaeal 16S rRNA gene copy numbers observed in our study were linked. This hypothesis, however, needs to be proven in future studies with the help of a more detailed analysis of the ammonia-oxidising microbial community and the presence of relevant functional genes (AOA and AOB amoA).

As this link was absent at the 5% application rate, bacterial nitrifiers are likely to have been selected, with any nitrified NH_4 being converted to NO_3. This was evidenced by the absence of nitrite in any of the 5% frass samples (Table 6) and the higher bacterial 16S rRNA gene copy numbers in the 5% compared to the 2.5% frass regimes, which was particularly marked where 5% BSFF was applied (Fig. 1). Why the observed nitrification "bottleneck" causing remarkably high soil nitrite concentrations (Table 6) only occurred in the 2.5% BWF and MWF regimes is not clear. Perhaps the nitrite build-up was caused by its oxidation being inhibited by free ammonia or nitrous acid (Taylor et al., 2019), analyses of which would be pertinent to future studies of frass-amended soil.

It should be acknowledged that microbial biomass C determination via chloroform fumigation extraction was not applicable in this soil at these high frass application rates due to the extremely high amounts of C detectable in the non-fumigated samples, even after pre-extraction. The DNA extraction results we report may be biased by DNA arising from worms or either the exoskeletons or digestive tracts of insects. For example, Eo et al. (2017) surmised amendment by rhinoceros beetle frass to have caused considerable enrichment of larval gut bacteria in the soil. While we could not distinguish soil microbial and insect DNA, the bias was consistently present in all experimental vessels and we assume that the vast majority of extracted DNA was from soil microbes. The facts that all frass treatments stimulated significant increases of saprotrophic fungi (Fig. 2) and, even in a heavy metal-contaminated substrate, soil microbial biomass as determined by CFE (Fig. 4), would attest to this. It should also not be overlooked that our results cannot be extrapolated to the field without consideration of what Jacquiod et al. (2013) termed the "microcosm effect". There may be, for example, exaggerated biostimulation of microbes benefitting from breakdown products of the chitin component of frass in closed experimental vessels kept in optimal temperatures.

With the exception of MWF at the 2.5% application rate, all frass treatments caused greater growth of saprotrophic fungi (expressed in extractable ergosterol contents) than in the vermicompost and control regimes (Fig. 2). This figure also illustrates that higher frass application rates led to a fungal competitive advantage. Extractable ergosterol contents, with the doubling of frass amendment, increased by a factor of at least four. The increase was not as marked in the bacterial 16S rRNA gene copy numbers, while the archaeal 16S rRNA gene copy numbers fell (Fig. 1). Fungal stimulation by frass application in soil was also reported by Fielding et al. (2013) in soil receiving 2% grasshopper frass. Lovett and Ruesink (1995) described a "visible mat of fungal hyphae" in soil samples amended with gypsy moth frass, albeit at a much higher rate (>20%) than in our study. Fungal growth was not reported to be increased by vermicompost application to soil by Lazcano and Domínguez (2011), in contrast to our results (Fig. 2). However, these authors observed stimulated bacterial growth following vermicompost amendment, as did we (Fig. 1).

Fig. 3, which expresses the ergosterol C as a percentage of the DNA carbon, depicts the stimulation of the fungal proportion of the microbial biomass at the 5% frass application rates. The relative increase in the fungal proportion of microbial biomass is much more marked when considering the difference between the 2.5 and 5% application rates for MWF and BWF. This may also explain the observed higher microbial biomass specific respiration rates in these regimes (Table 5). Increased fungal dominance has been observed to result in lower carbon use efficiency by Iqbal et al. (2016).

4.2. *Heavy metal toxicity mitigation experiment*

Frass amendments significantly reduced 0.01 M $CaCl_2$-extractable Cd, Cu, Zn and Ni in a heavy-metal spiked, C-poor substrate relative to the non-amended control (Fig. 5). This phenomenon is most likely to have arisen as a combination of functional groups in the frass sorbing or

complexing the metals, as has been described in the use of organic matter addition as an ameliorative strategy (Wuana and Okieimen, 2011). Vermicompost was effective only in significantly reducing the extractable Cu relative to the control. This is likely to demonstrate Cu's marked affinity for humic substances, which are significant components of vermicompost (Muscolo et al., 1999).

Nickel, cadmium and zinc's interaction with humic components is less pronounced. In acidic substrates such as the one used in our experiment, their sorption and hence bioavailability is greatly affected by pH (Kabata-Pendias, 2011). The pH-rising effect of amending the relatively low pH (5.48) substrate in our study with frass, which were all approximately ten times more alkaline (Table 4), would have increased the proportion of pH-dependent negative sites to which the heavy metal cations could sorb.

Soil microbial biomass is considered a useful indicator when assessing heavy metal toxicity but is subject to heterogeneity due to the "patchiness" of heavy metal and C distribution in soil (Chander et al., 2001; Giller et al., 2009). Microbial biomass C was significantly higher in BSFF and BWF-amended soil samples than in the non-amended control; vermicompost had no such effect (Fig. 4). The frass-induced increase in microbial biomass C was possibly linked to improved energy provision. The labile C fraction of the amendments could have served as an energy source and thereby stimulated microbial growth. Another possible factor influencing microbial biomass C might have been the provision of additional nutrients by the frass amendments. With the exception of NO_3 and Ca, all frass types contained significantly higher extractable nutrients than did vermicompost (Tables 2 and 3), although this effect is likely to have been masked by the nutrient solution additions. With the aforementioned effect of frass application increasing the proportion of sorption sites, their growth promoting effects on microbial biomass were likely to have been a combined result of reduced heavy metal availability, nutrient provision and enhanced energy supply.

5. Conclusions

All frass types featured high extractable concentrations of macro- and micronutrients. These amendments stimulated microbial C and N mineralisation. Where 2.5% BWF or MWF was applied, a nitrification 'bottleneck' became apparent with a build-up of considerable nitrite concentrations and an increase in archaeal 16S rRNA gene copy numbers. Whether this nitrite build-up was caused by an increase in ammonia-oxidising archaea needs to be verified with more specific qPCR analyses in future studies. At the 5% application rate, BSFF stimulated overall microbial growth. MWF and BWF did not. This was possibly an artefact of their contrasting extractable nutrient contents or short term C limitation caused by excess N.

Frass application significantly reduced extractable concentrations of heavy metals in a substrate artificially contaminated with Zn, Cd, Cu and Ni. This was likely due to functional groups in the frass sorbing or complexing the metals and frass application increasing the proportion of the substrate's negatively charged sorption sites. BSFF and BWF induced significantly higher microbial biomass in the substrate relative to the non-amended control. This arose as a combination of reduced metal bioavailability, nutrient provision and enhanced energy supply.

All frass types seem suitable as ameliorative amendments to heavy metal-contaminated soils. Of the three, only BSFF would appear to be suitable as an organic fertiliser due to its stimulation of the microbial biomass at both application rates without measurable nitrite build-up. However, the robustness of these conclusions will only be known after the influence of larval feedstock and the possible impacts of frass sterilisation have been fully understood.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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